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LruA and LruB, Novel Lipoproteins of Pathogenic *Leptospira interrogans* Associated with Equine Recurrent Uveitis

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Recurrent uveitis as a sequela to *Leptospira* infection is the most common infectious cause of blindness and impaired vision of horses worldwide. Leptospiral proteins expressed during prolonged survival in the eyes of horses with lesions of chronic uveitis were identified by screening a phage library of *Leptospira interrogans* DNA fragments with eye fluids from uveitic horses. Inserts of reactive phages encoded several known leptospiral proteins and two novel putative lipoproteins, LruA and LruB. LruA was intrinsically labeled during incubation of *L. interrogans* in medium containing [^{14}C]palmitic acid, confirming that it is a lipoprotein. *lruA* and *lruB* were detected by Southern blotting in infectious *Leptospira interrogans* but not in nonpathogenic *Leptospira biflexa*. Fractionation data from cultured *Leptospira* indicate that LruA and LruB are localized in the inner membrane. Uveitic eye fluids contained significantly higher levels of immunoglobulin A (IgA) and IgG specific for each protein than did companion sera, indicating strong local antibody responses. Moreover, LruA- and LruB-specific antisera reacted with equine ocular components, suggesting an immunopathogenic role in leptospiral uveitis.

Leptospirosis is a zoonosis caused by pathogenic species of *Leptospira* that affects humans, wildlife, and many domesticated animals. The disease in humans varies from a mild flulike form to a more severe syndrome involving multiorgan failure, whereas in horses the infection is mainly associated with spontaneous abortion and recurrent uveitis. Equine recurrent uveitis (ERU), also known as moon blindness or periodic ophthalmia, is a major cause of blindness in horses and is characterized by episodes of intraocular inflammation that develop weeks to months after an initial uveitic episode and recur at regular intervals (12). *Leptospira interrogans* serovar Pomona and *Leptospira kirschneri* serovar Grippotyphosa have been incriminated as the most common infectious causes of the disease in North America and Europe, respectively (19, 21). The association of ERU with pathogenic leptospires has been established by high titers of leptospiral agglutinins in the blood and aqueous humor (19) and by isolation of *Leptospira* from ocular fluids of uveitic horses (5, 9, 21). Typically, ERU appears as a late sequela of leptospiral infection that generally appears months to years after a naturally acquired or experimentally induced infection (33, 42, 47).

ERU is widely considered to be an immune-mediated disease, and eyes with ERU exhibit infiltration of lymphocytes, plasma cells, and macrophages into the ciliary body and iris, thereby constituting morphological evidence of breach of immune privilege. $CD4^+$ T lymphocytes are the most abundant infiltrating cells in the anterior uveal tracts of uveitic horses. The T-lymphocyte response in such horses has a Th1 bias based on quantitative reverse transcription-PCR (RT-PCR), which showed significantly greater interleukin-2 (IL-2)/gamma interferon- than IL-4-specific mRNA (11). Also, peripheral blood leukocytes of chronically uveitic horses do not exhibit a Th1 response, consistent with an independent local response (11).

Pathogenic *Leptospira* spp. respond to environmental stimuli such as temperature (34), osmolarity (32), and other, unknown cues in the body of the host (1, 32, 37) by altering expression of many proteins. The eye, which is filled with a very dilute aqueous solution of albumin, chloride, bicarbonate, neutral amino acids, and small amounts of insoluble proteoglycans, poses unique challenges to the adaptability of *Leptospira* to a nutrient-poor environment (10). Design of effective therapies for management of the uveitis is dependent upon an understanding of how *Leptospira* spp. survive in the eye and initiate pathological changes. Although there is well documented evidence of an association of infection with *Leptospira* and ERU, the pathogenesis of the resulting uveitis is largely unknown. One reason for this is a lack of information regarding antigenic leptospiral proteins expressed during uveitis. The present study was undertaken to identify leptospiral proteins expressed during ocular infection and has led to the identification of two novel immunoreactive lipoproteins with possible roles in ERU pathogenesis.

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TABLE 1. Histopathology and serology of uveitic eyes

Eye no.	Pathology of anterior chamber, iris-ciliary apparatus, lens, vitreous, and retina[a]											MAT titer[b]	ELISA OD_{490}[c]
	ASY	C/PAC	LPI	IRE	AMY	CCT	CMN	LRU	CPV	LPR	RDE		
U14	−	++	+++	++	+/−	+++	+/−	−	++	−	++	1/51,200	>3.0
U16	++	+	+	−	−	+++	+++	+++	+++	−	++	1/102,400	>3.0
U17	++	−	+	++	−	+++	+++	−	++	+++	+++	1/204,800	>3.0
U19	++	+	+	+	++	++	++	+	+++	+/−	+++	NA[d]	>3.0
U20	+	+	++	++	++	++	++	+	++	+/−	++	NA	>3.0

[a] +, mild; ++, moderate; +++, severe; −, negative; ASY, anterior synechia; C/PAC, cells/proteins in anterior chamber; LPI, lymphoplasmocytic iridocyclitis; IRE, iris edema; AMY, amyloid deposition (iris); CCT, cataract; CMN, cataract and mineralization; LRU, lens rupture; CPV, cells or proteins in posterior chamber; LPR, lympho plasmacytic retinitis; RDE, retinal detachment.
[b] *Leptospira interrogans* serovar Pomona.
[c] JEN4 sonicate (100 ng/well); eye fluid dilution, 1:400. OD_{490}, optical density at 490 nm.
[d] NA, not available.

MATERIALS AND METHODS

***Leptospira* culture.** *Leptospira interrogans* serovars Pomona type kennewicki (JEN4), Pomona (Pomona) Copenhageni (M 20), Canicola (Hond Utrech IV), Grippotyphosa (Andaman), Hardjo (Hardjoprajitno), and Bratislava (Jez Bratislava) were kindly provided by Mike Donahue (Livestock Disease Diagnostic Center, University of Kentucky, Lexington). *Leptospira biflexa* serovar Biflexa was obtained from The National Veterinary Services Laboratories, Ames, Iowa. Leptospires were grown in Johnson-Harris bovine serum albumin–Tween 80 medium (Bovuminar PLM-5 Microbiological Media; Intergen, Purchase, NY) at 30°C unless otherwise indicated.

Eye fluids and eye tissue extracts. Eye fluids and companion sera from horses of varied age, breed, and origin were obtained from a commercial horse slaughter plant in North America. Eyes with gross evidence of uveitis were enucleated after slaughter, and aqueous humor was removed with a 10-ml syringe and stored at −20°C. The eyes were placed in 10% formaldehyde for subsequent embedding, sectioning, and staining with hematoxylin and eosin for histologic examination. Eye fluids and sera were assayed for antibodies to serovars Pomona, Canicola, Icterohemorrhagiae, Hardjo, Bratislava, and Grippotyphosa in the microscopic agglutination test (MAT) (Table 1). Extracts were prepared from the ciliary body, cornea, lens, and retina of a normal eye from a young horse serologically negative for *Leptospira* (38).

Library screening and plasmid rescue. A lambda ZAP II library containing 3- to 5-kb fragments of *L. interrogans* serovar Pomona type kennewicki DNA (23) was screened to identify phage expressing gene products reactive with pooled eye fluids from uveitic horses. Following propagation on *Escherichia coli* XL-1 MRF′ (Stratagene, La Jolla, CA) lawns, plaques were transferred in duplicate to IPTG (isopropyl-β-D-thiogalactopyranoside)-saturated nitrocellulose disks and immunoblotted with pooled eye fluids, diluted 1:600, from five uveitic horses (Table 1). The secondary antibody was horseradish peroxidase (HRP)-labeled protein G (Zymed, San Francisco, CA) diluted 1:4,000. Immunopositive plaques were identified by using 4-chloro-1-naphthol as the substrate. Positive plaques on agar plugs were transferred to 500 μl of SM buffer and allowed to elute overnight at 4°C. Reactive plaques were rescreened until clonal. Plasmids containing inserts of leptospiral DNA were rescued from selected reactive phages by using ExAssist helper phage and *E. coli* SOLR (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

DNA sequencing and analysis. Plasmid DNA was isolated using a QIAprep spin miniprep kit (QIAGEN, Valencia, CA) and sequenced in a commercial sequencing facility (Davis Sequencing LLC, Davis, CA) using T3, T7, and custom-designed primers (Table 2). Sequences were edited and connected using Chromas 1.61 (Technelysium Pty. Ltd., Queensland, Australia) and DNASIS (Hitachi Software Engineering Co., Ltd., San Francisco, CA). The sequences were compared with *L. interrogans* serovar Lai strain 56601 (41) and *L. interrogans* serovar Copenhageni Fiocruz L1-130 (36) genomic sequences at http://www.tigr.org/. Analyses of nucleotide and deduced amino acid sequences were performed using DNASIS, the Genetics Computer Group package of programs (Wisconsin Package version 10.0; Genetics Computer Group, Madison, WI), PSORT (http://psort.nibb.ac.jp/), SignalP (4), LipoP (22), TMHMM (http://www.cbs.dtu.dk/), and COILS (http://www.ch.embnet.org/index.html). Homologies were identified by a BLAST search using the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

Cloning and expression. Primers a1-1F plus a1-1R and b5-1F plus b5-1R (Table 2), specific for of *lruA* and *lruB*, respectively, were designed using Primer 2 (Scientific & Educational Software, 1991). Following PCR amplification of chromosomal DNA of *L. interrogans* serovar Pomona type kennewicki (JEN4), amplicons were digested with BamHI and XhoI and inserted into pET-15b (Novagen, Madison, WI) predigested with the same restriction endonucleases. Recombinant plasmids were transformed into *E. coli* BL21(DE3) (Novagen, Madison, WI). Expression of His_6-LruA and His_6-LruB was induced with 1 mM IPTG when cultures reached an optical density of 0.6 at 600 nm, and cells were harvested after 2 to 3 h. Recombinant His-tagged proteins were isolated using Talon metal affinity resin (Clontech Laboratories, Inc.) in buffer containing 8 M urea according to the manufacturer's recommendations. The purity of recombinant proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). His_6-LruA and His_6-LruB were dialyzed against 10 mM Tris (pH 7.5) containing 50 mM NaCl.

[^{14}C]palmitate radiolabeling and immunoprecipitation of native LruA. A 12-ml culture of *L. interrogans* Fiocruz L1-130 containing 2×10^8 cells per ml in the log phase of growth was mixed with 50 μCi of [U-^{14}C]palmitic acid (Amersham, Piscataway, NJ) and incubated at 30°C until the density of the bacteria reached 1.3×10^9/ml; 1.2×10^{10} bacteria were collected by centrifugation for 3 min at 9,000 × *g* in a microcentrifuge and frozen at −20°C. The bacteria were lysed by suspending the pellets combined in a single microcentrifuge tube with 1.2 ml boiling lysis buffer (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 2 mM EDTA, and 0.2% SDS) and boiling for 5 min. The lysate was subjected to centrifugation for 10 min at 4°C at 14,000 × *g*, and the insoluble material was removed. For immunoprecipitations, 795 μl immunoprecipitation buffer (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.2% Triton X-100) was mixed with 200 μl lysate (representing 2×10^9 bacteria) and 5 μl anti-LipL41 or anti-LruA rabbit serum and incubated on ice overnight. Twenty-five microliters of EZ View Red protein A affinity gel (Sigma) was then added, and the mixture was mixed for 2 h at 4°C. The antibody-antigen complexes bound to protein A were recovered by centrifugation at 15,000 × *g* for 8 s, washed twice with 800 μl immunoprecipi-

TABLE 2. Primers used in this study

Gene and primer	Sequence
iruA	
a1-1F	5′-GCG CTC GAG ATT GAG GAA TTA AGT GAT GC-3′
a1-1R	5′-GCG GAT CCA GGA TCG TCT TTA CTC TCA G-3′
a1-1uF	5′-ATC GCG AAA CTC ATC GAG GAG CAA-3′
a1-1uR	5′-TTC TTT CGG CCA ACT CAG TGG CTT-3′
a1-1dF	5′-AGC AGC TGA AGA ATC GAG AGT AGC-3′
a1-1dR	5′-TGA GCG TGA ATC TGG ATG AAG AGA AC-3′
a1rt-F	5′-ACA AGA GCT AAG TCT GCA GG-3′
a1rt-R	5′-AGC AAG CTG AGA AGC ATA GG-3′
iruB	
b5-1F	5′-GCG CTC GAG AAC GAC TCA GAA CGT TTA GC-3′
b5-1R	5′-GCG GAT CCC TTA AAT TGA AAA GTC CGT G-3′
b5-1uF	5′-ATT CCG CCA AAT CAA CGT AGG TGC-3′
b5-1uR	5′-ATA AGT GAC CGC TGC GTC AGG ATT-3′
b5-1dR	5′-CAT GAT CGC CAG TTC TTG GTT GTT G-3′
b51rt-F	5′-ACT GCG ACA AGA GCT CAA GT-3′
b51rt-R	5′-GTT GTC TAT CGG TCC AGA TG-3′

tation wash buffer (10 mM Tris-Cl, pH 8.0, 0.4 M NaCl, 0.01% Triton X-100), and washed once with 800 μl no-salt wash buffer (10 mM Tris-Cl, pH 8.0, 0.01% Triton X-100). Pellets were resuspended with 100 μl final sample buffer containing 0.25 M phenylmethylsulfonyl fluoride. Ten microliters of each sample was subjected to electrophoresis (12% PAG-SDS gel; Cambrex), and bands were detected by fluorography following a 30-min treatment of the gel with Amplify (Amersham, Piscataway, NJ).

Detection of genes in different *Leptospira* spp. and serovars. DNAs of *L. interrogans* serovars Pomona, Canicola, Grippotyphosa, Hardjo, and Bratislava; *L. biflexa* serovar Biflexa; *Leptospira weilii* (Sarmin); *Leptospira inadai* (LT430); and *Leptonema illini* (Illini 3055) were isolated from 5-ml cultures as previously described (1). Leptospiral DNAs were digested overnight with HindIII at 37°C. Digested DNAs were separated on a 0.8% agarose gel for 4 h at 50 V, transferred to a Hybond-N nylon membrane (Amersham, Piscataway, N.J.), and fixed by UV cross-linking according to the manufacturer's protocol.

Primers a1-1F plus a1-1R and b5-1F plus b5-1R (Table 2), specific for of *lruA* and *lruB*, were used in the PCR to amplify the *lruA* and *lruB* genes and were labeled with digoxigenin by using the DIG High Prime DNA labeling and detection kit (Roche Applied Science, Indianapolis, IN). Prior to digoxigenin labeling, the *lruA* PCR amplicon was digested with HindIII and the larger fragment extracted from the gel. The UV-cross-linked nylon membrane was subjected to prehybridization at 42°C for 30 min in DIG Easy Hybridization solution. After denaturation, approximately 25 ng/ml of probe was mixed with DIG Easy Hybridization solution and incubated with the membrane at 42°C with gentle agitation. The next day, the membrane was washed for 15 min at room temperature with three changes of the buffer containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS and was then washed thrice for 15 min at 65°C in 0.5× SSC with 0.1% SDS, prewarmed to 65°C. After stringency washes, the membrane was treated with anti-digoxigenin–alkaline phosphatase, followed by a chemiluminescent substrate (DIG High Prime DNA labeling and detection kit). Hybridization was detected by exposing the membrane to an X-ray film (Pierce, Rockford, IL).

SDS-polyacrylamide gel electrophoresis and immunoblotting. SDS-PAGE was performed in a 12% acrylamide gel using a discontinuous buffer system as described elsewhere (27). Samples were mixed with an equal volume of 2× sample loading buffer containing 125 mM Tris-Cl, 4% SDS, 2% glycerol, 1% β-mercaptoethanol, and 0.5% bromophenol blue and boiled for 5 min before loading. Electrophoresis was carried out in an X-Cell SureLock minicell (Invitrogen, Carlsbad, CA) for 2 h at 125 V in Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Proteins were transferred to Protran nitrocellulose membranes (0.2-μm pore size; Schleicher & Schuell, Keene, NH) and blocked with 4% nonfat dry milk in Tris-buffered saline (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5). Membranes were incubated with protein-specific antiserum, eye fluid, or antiserum to eye antigens, followed by incubation with protein G conjugated to horseradish peroxidase (Zymed, San Francisco, CA). Membranes were developed with the ECL Western blot detection system (Amersham, Piscataway, NJ), and bands were visualized with Hyperfilm (Amersham) or by using 4-chloro-1-naphthol (Sigma, St. Louis, MO).

RT-PCR. Total RNA was extracted from *L. interrogans* serovar Pomona type kennewicki grown at 30°C and 37°C by using the RNeasy minikit (QIAGEN, Valencia, CA). RT-PCR was performed with gene-specific primers a1rt-F, a1rt-R, b51rt-F, and b51rt-R (Table 2) on total RNA, using the SuperScript one-step RT-PCR kit (Invitrogen, Carlsbad, CA). cDNA synthesis was accomplished in 30 min of incubation at 50°C followed by denaturation at 94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 70°C for 1 min; and a final extension step at 72°C for 5 min.

Enzyme-linked immunosorbent assay (ELISA). A checkerboard titration was performed in flexible 96-well flat-bottom polystyrene MicroTest III assay plates (Falcon; Becton Dickinson, Oxnard, CA) with pools of uveitic and normal eye fluids diluted 200-, 400-, 600-, 800-fold and tested against 25, 50, 100, and 200 ng/well of antigen to determine the optimum concentrations of recombinant LruA and LruB. Wells were coated with 100 ng protein, followed by blocking with 4% nonfat dry milk. Eye fluids (1:600) or serum (1:400) was added and left for 1 h at 37°C. Bound immunoglobulin G (IgG) was detected using HRP-protein G (Zymed, San Francisco, CA) and IgA with α-chain-specific monoclonal antibody (BVS-2) followed by HRP conjugated goat anti-mouse. Plates were developed with *o*-phenylenediamine (Sigma, St. Louis, MO). Analysis of the data was performed using Student's *t* test and analysis of variance.

Polyclonal antisera. Polyclonal antisera were raised in New Zealand White rabbits by subcutaneous administration of 1 μl of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Sigma, St. Louis, MO) and 100 μg of recombinant protein adsorbed to aluminum hydroxide (Alhydrogel; Accurate Chemical & Scientific Corp., Westbury, NY). Booster injections contained 100 μg (subcutaneous) and 5 μg (intravenous) of the antigen and were administered 14 and 28 days after the primary immunization. Serum was obtained 35 days after the primary immunization (20).

Membrane solubilization with Triton X-114. The outer membrane fraction of low (fourth)-passage *L. interrogans* serovar Copenhageni was extracted by Triton X-114 solubilization and phase partitioning as described previously (14). Briefly, leptospires washed in phosphate-buffered saline containing 5 mM $MgCl_2$ were extracted in 0.5% protein-grade Triton X-114 (Calbiochem), 150 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA at 4°C. Insoluble material (protoplasmic cylinder) was pelleted by centrifugation at 10,000 × *g* for 10 min. Phase separation of the supernatant was performed by warming it to 37°C after the addition of 20 mM $CaCl_2$, followed by centrifugation for 10 min at 1,000 × *g*. Proteins in aqueous and detergent phases were precipitated with acetone.

Nucleotide sequence accession numbers. Nucleotide sequences deposited in the GenBank database have accession numbers AY741529 (*lruA*) and AY741530 (*lruB*).

RESULTS

Identification and analysis of *lruA* and *lruB*. Screening of approximately 10^5 plaques of a lambda library of *L. interrogans* serovar Pomona type kennewicki with pooled eye fluids from uveitic horses revealed 14 reactive plaques. Plasmids rescued from these phages were sequenced and compared with *L. interrogans* serovar Lai strain 56601 (41) and *L. interrogans* serovar Copenhageni Fiocruz L1-130 (36) genomic sequences. Homologies to eight different regions of the *L. interrogans* genome were demonstrated. These regions encode the previously described leptospiral proteins LigA/LigB (31, 37) (four phagemids), LigC (one phagemid), GrpE/DnaK/DnaJ (2) (four phagemids), and Qlp42 (30, 35) (two phagemids), plus two novel proteins. Phagemids pA1, pD1, and pB5, which encode these two proteins, were selected for further analysis.

Sequencing of pA1, pD1, and chromosomal DNA of JEN4 using primers T3, T7, a1-1uF, a1-1uR, a1-1dF, and a1-1dR (Table 2) revealed an open reading frame positioned in a 2,243-bp fragment of JEN4 chromosomal DNA (GenBank accession no. AY741529). This open reading frame encoded a protein (designated LruA) of 555 amino acids with a predicted molecular mass of 62 kDa. The amino terminus of LruA consisted of a 22-amino-acid signal sequence with a putative lipobox (FIS ↓ C) at its carboxy terminus. Several hexanucleotides resembling the −10 region of the σ^{70} bacterial promoter were present upstream of *lruA*. Downstream of this gene, a stem-and-loop structure ($\Delta G = -18.6$ kcal/mol) followed by a stretch of five thymines resembled a ρ-independent transcriptional terminator. LruA also contained a conserved domain, LysM, between residues 406 and 461. This domain has been found in a variety of microorganisms and is apparently associated with peptidoglycan binding (3, 29). LruA showed 99.6% (two amino acid substitutions) and 99.8% (one amino acid substitution) identity with LIC11003 of *L. interrogans* serovar Copenhageni (36) and LA3097 of *L. interrogans* serovar Lai (41), respectively.

Phagemid pB5 contained a 1,936-bp insert (GenBank accession no. AY741530) that encoded two proteins, pL13 and LruB. Analysis of the nucleic acid sequence demonstrated ribosomal binding sites for both genes but a putative promoter for *pL13* and a transcriptional terminator for *lruB* only, suggesting translation of pL13 and a 48-kDa protein, LruB, from a polycistronic mRNA. The N-terminal 22 amino acid residues of LruB resembled a signal peptide sequence with a potential lipoprotein signal peptidase II cleavage site, FSN ↓ C. A con-

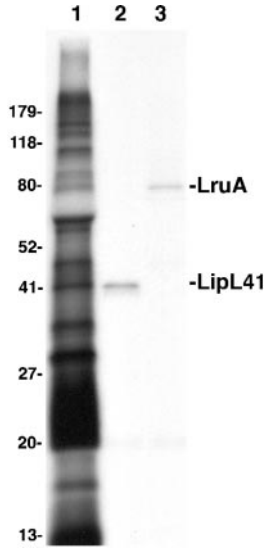

FIG. 1. LruA is acylated by *L. interrogans*. An autoradiogram of whole *L. interrogans* and immunoprecipitated LipL41 and LruA after intrinsic labeling with [^{14}C]palmitate and separation by SDS-PAGE is shown. Lane 1 contains *L. interrogans* labeled with [^{14}C]palmitate, including lipopolysaccharide (<27 kDa) and lipoproteins (>27 kDa). Lanes 2 and 3 contain material immunoprecipitated by addition of LipL41 and LruA antisera, respectively, to a total membrane lysate of *L. interrogans*. Each lane contains material from 2×10^8 *L. interrogans* organisms. Locations of molecular size standards are shown in kilodaltons on the left.

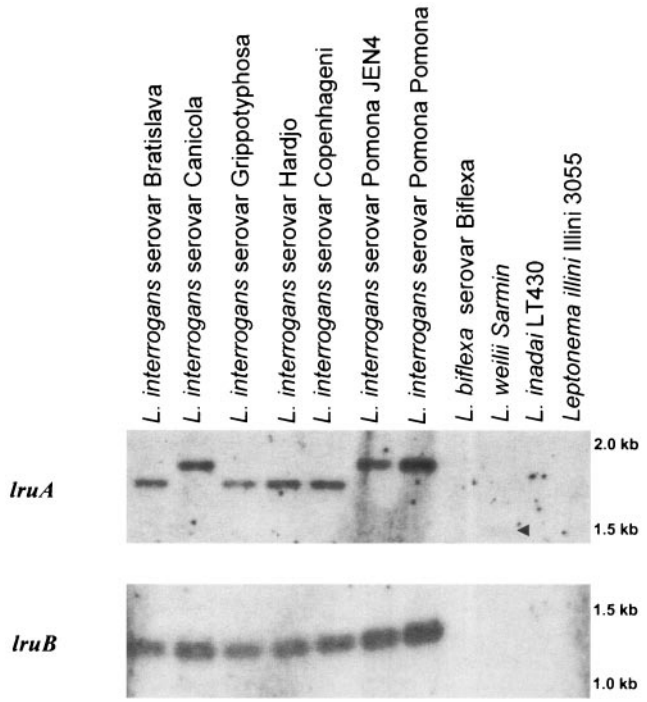

FIG. 2. Detection of *lruA* and *lruB* sequences in *Leptospira* spp. Detection of *lruA* and *lruB* sequences by Southern blotting in pathogenic serovars of *L. interrogans* or other *Leptospira* spp. is shown; *Leptonema illini* and *L. interrogans* serovar Pomona JEN4 were included as controls. The arrowhead indicates the *lruA* sequence in *L. weilii*.

served domain, IrpA (PSSM-Id 12815), spanning from Q_{42} to T_{351} was predicted for LruB. Proteins with this conserved domain have been described for several bacteria (24, 29) and are apparently associated with iron metabolism regulation. LruB showed 94.1% (25 amino acid substitutions) and 97.7% (10 amino acid substitutions) identity with LA3469 of *Leptospira interrogans* serovar Lai (41) and LIC10713 of *Leptospira interrogans* serovar Copenhageni (36), respectively. These amino acid substitutions were clustered between residues 155 and 176 for LIC10713 of *Leptospira interrogans* serovar Copenhageni and from amino acid residues 155 to 182 and 216 to 236 for LA3469 of *Leptospira interrogans* serovar Lai.

Acylation of LruA. Intrinsic labeling of *L. interrogans* with [^{14}C]palmitate resulted in acylation of lipopolysaccharide and previously identified lipoproteins, including LipL32 (16), LipL36 (15), and LipL41 (43). Only some of the bands observed by Coomassie blue staining of the gel were labeled with [^{14}C]palmitate, thus confirming the selectivity of the procedure (data not shown). Because LruA is a leptospiral inner membrane protein, immunoprecipitation of LruA was performed with total membrane lysate rather than with Triton X-100 extract (15, 16, 43). Immunoprecipitation of the total membrane lysate of [^{14}C]palmitate-labeled *L. interrogans* by using LruA antiserum confirmed that LruA is acylated by *L. interrogans* (Fig. 1). Antiserum to LipL41 was included as a positive control for immunoprecipitation.

Distribution of *lruA* and *lruB* among *Leptospira* spp. The distribution of *lruA* and *lruB* in a number of pathogenic and saprophytic strains of *Leptospira* was examined by Southern blotting. *lruA* was found in *L. interrogans* serogroup Icterohemorrhagiae serovar Copenhageni and serovars Pomona (strains pomona and JEN4), Canicola, Hardjo, Bratislava, and Grippotyphosa and in *L. weilii* but not in *L. biflexa*, *L. inadai*, or *Leptonema illini* (Fig. 2). *lruB* was also found to be present in all *L. interrogans* serovars but not in saprophytic *L. biflexa*, the intermediate pathogen *L. inadai*, or the non-*L. interrogans* pathogenic *L. weilii*. (Fig. 2). *lruA* and *lruB* appear to be restricted to pathogenic *Leptospira* species, since they were not detected in saprophytic *L. biflexa*. *lruB* seems to be present only in *L. interrogans* and not in other species (Fig. 2).

Expression and cellular localization of LruA and LruB. Since culture temperature can affect expression of several *L. interrogans* genes, transcription of *lruA* and *lruB* in *L. interrogans* serovar Pomona grown at 30°C or 37°C was examined by RT-PCR. *lruA* and *lruB* transcripts were clearly detectable in cultures grown at 30°C or 37°C (data not shown). Reactions without reverse transcriptase yielded no product, indicating purity of the RNA preparations. Immunoblotting of whole-cell lysates from cultures grown at 30°C or 37°C with LruA- and LruB-specific rabbit antisera showed equivalent expression levels of each protein (data not shown).

Triton X-114 was used to separate hydrophobic outer membrane proteins, hydrophilic periplasmic proteins, and the protoplasmic cylinder (Fig. 3), using antisera to known outer and inner membrane proteins LipL32 and LipL31, respectively (16, 18). Triton X-114 fractions immunoblotted with antisera specific for LruA and LruB revealed a pattern of fractionation similar to that of LipL31 in both cases. This indicated that

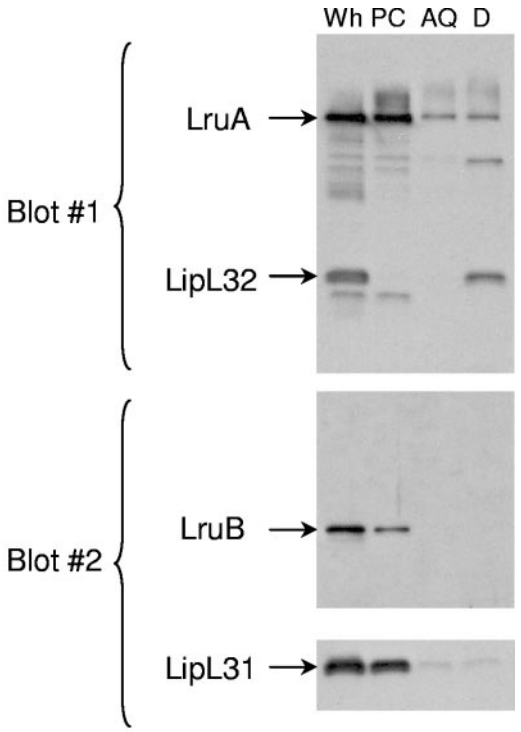

FIG. 3. Cellular localization of LruA and LruB. Immunoblots of Triton X-114 extracts of *L. interrogans* show the cellular localization of LruA and LruB. Blot 1 contains whole cells (Wh), Triton X-114-insoluble material (PC), aqueous phase (AQ), and detergent phase (D) and was probed with rabbit antisera to LipL32 (1:20,000) and LruA (1:1,000). Blot 2 was probed with rabbit antisera to LruB (1:500) and LipL31 (1:10,000). Antisera to LipL32 and LipL31 were included as controls for proteins of detergent and protoplasmic cylinder fractions, respectively.

LruA and LruB are found largely, if not exclusively, in the leptospiral inner membrane.

LruA- and LruB-specific antibody levels in sera and eye fluids of uveitic horses. Eye fluids and sera from uveitic horses and sera from aborted mares were tested by ELISA, using LruA and LruB as antigens (Fig. 4). High levels of LruA- and LruB-specific IgG and IgA antibodies were detected in uveitic eye fluids but not in companion sera (Fig. 4). Aborted mares with high serum MAT titers showed only moderate serum levels of LruA- and LruB-specific IgG. IgA specific for LruA and LruB was detected only in uveitic eye fluids. Normal eye fluids did not contain detectable levels of LruA- and LruB-specific IgG and IgA antibodies (not shown).

Cross-reactivity of LruA and LruB with equine ocular tissue. Possible roles of LruA and LruB in autoimmunity were investigated by immunoblotting extracts of ciliary body, lens, and retina of a normal equine eye with specific antisera. LruA antiserum reacted with a ~22-kDa band in lens extract and a ~65-kDa band in ciliary body extract (Fig. 5A and B, respectively). Antiserum to LruB reacted very strongly with a ~30-kDa band of equine retinal extract (Fig. 5C). Preimmunization rabbit sera served as controls for nonspecific reactivity. Antiserum to Lk73.5 (1), an immunoreactive, host-inducible sphingomyelinase of *L. interrogans*, showed no reactivity with eye tissue extracts (not shown).

DISCUSSION

Recurrent uveitis is a well established sequela of natural and experimentally induced *Leptospira* infection in the horse and typically appears months to years following natural or experimental exposure (8, 42, 47). Culture of the organism from uveitic fluids indicates an ability to adapt to conditions in aqueous and vitreous humors. Compared to serum, with which it is isosmolar, aqueous humor contains an excess of chloride, bicarbonate, ascorbate, lactate, and neutral amino acids (10). Our study for the first time provides information on leptospiral proteins expressed in this environment and which stimulate local antibody responses. In this study, screening of an expression library using eye fluids from uveitic horses identified a number of known and novel lipoproteins. Here we describe LruA and LruB, novel immunogenic lipoproteins of *Leptospira* that are expressed in the eyes of uveitic horses and cross-react with equine ocular tissue.

LruA and LruB have Phe_{-3}-Ile_{-2}-$Ser_{-1} \downarrow Cys_{+1}$ and Phe_{-3}-Ser_{-1}-$Asn_{-1} \downarrow Cys_{+1}$ as potential signal peptidase cleavage sites, respectively, which conform to the consensus lipobox sequence of spirochetal lipoproteins (17). [^{14}C]palmitate radiolabeling experiment confirmed that, in *L. interrogans*, the FIS $\downarrow$ C sequence in the LruA signal peptide is a lipoprotein signal peptidase cleavage site as predicted by the LipoP algorithm (22). This is the first experimental evidence that serine is allowed in the -1 position of leptospiral lipoproteins. We were unable to immunoprecipitate sufficient quantities of LruB to demonstrate palmitate labeling, possibly due to the low level of LruB expression or to degradation during immunoprecipitation. However, LruB is likely to be a lipoprotein, because the FSN $\downarrow$ C sequence in the LruB signal peptide is a predicted lipoprotein signal peptide cleavage site by the LipoP algorithm and because asparagine in the -1 position has been demonstrated for the leptospiral lipoprotein LipL41 (43).

Evidence for intraocular expression of both proteins is the much higher level of specific antibodies in uveitic ocular fluids than in companion sera. The higher ocular levels may be explained by local synthesis, an intact blood-eye barrier, and lack of degradation. The very low levels of specific IgG and IgA in companion sera suggest that systemic antibody responses to LruA and LruB in uveitic horses are suppressed or poorly expressed during the bacteremia that preceded ocular invasion. Lack of expression during infection is a less likely explanation of very low antibody levels in sera of uveitic horses, since LruA- and LruB-specific IgG levels are significantly ($P < 0.001$) higher in sera of recently aborted mares. The low levels are a manifestation either of a deviant systemic immune response or of a systemic response that has waned over the possibly long interval since systemic immune stimulation following initial infection. A previous comparison (46) of amounts and isotypes of immunoglobulin in uveitic and normal equine vitreous humor revealed significantly larger amounts of IgA in uveitic fluids and absence of IgM from normal and uveitic fluids. Amounts of IgGa, IgGb, IgGc, and IgG(T) in these fluids were about 1,000-fold less than those in companion sera but in the same proportions. It was therefore concluded that there is local IgA synthesis in uveitic eyes but that IgA and other immunoglobulins in normal fluids were derived from plasma. Although quantitation of IgG isotypes was not done

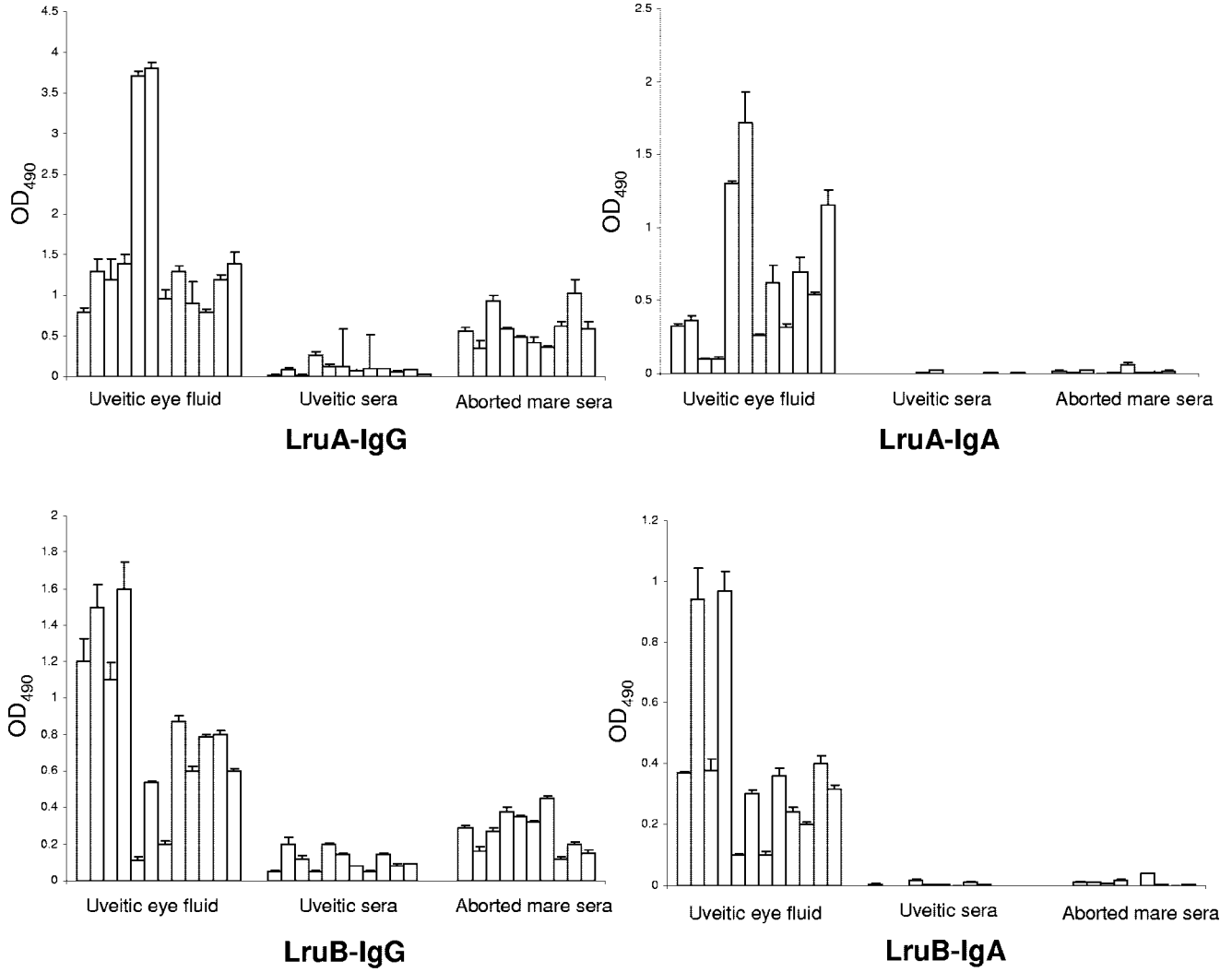

FIG. 4. LruA- and LruB-specific IgG (left panels) and IgA (right panels) levels in eye fluids and companion sera of 12 uveitic horses and in sera of 10 recently aborted mares. Error bars indicate standard deviations. OD_{490}, optical density at 490 nm.

for the uveitic fluids in the present study, the significantly ($P < 0.001$) lower levels of LruA- and LruB-specific IgG in companion sera indicate that local synthesis of IgG and not diffusion from plasma is the source of specific antibody in eye fluids of horses with ERU.

Prolonged intraocular survival of *Leptospira* spp. in the face of antibody responses to bacterial proteins indicates an absence of cells and molecules involved in the innate immune response and bacterial clearance. This may be explained by the immunosuppressive and anti-inflammatory effects of transforming growth factor β2 and other factors responsible for ocular immune privilege (13, 44, 45). Although unproven, it is likely that *Leptospira* spp. which enter the ocular compartment induce anterior chamber-associated immune deviation (ACAID), an important manifestation of immune privilege resulting in an inability of the host to display delayed hypersensitivity reactivity to *Leptospira* antigens and to produce *Leptospira*-specific complement-fixing antibodies (25, 26). A third potential outcome of ACAID is the induction and differentiation in the spleen of regulatory T cells that suppress *Lepto-*

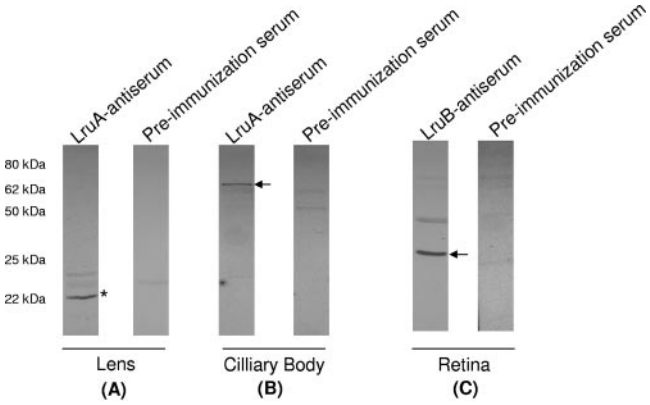

FIG. 5. Cross-reactivity of LruA and LruB with equine ocular tissue. (A and B) Immunoblots showing reactivity of rabbit antiserum to LruA with extracts of normal equine lens and ciliary body. (C) Reactivity of LruB antiserum with extracts of normal equine retina. Preimmunization sera from rabbits were used as controls for specificity of binding. The arrows and asterisk indicate cross-reactive bands to LruA- or LruB-antiserum in normal equine ocular tissues.

spira-specific Th1 and Th2 responses. Once induced, ACAID is long lasting, and so its effects would be expected to persist into the later stages of ERU.

Although direct *Leptospira*-mediated injury to the eye structure cannot be ruled out in explaining the pathogenic mechanisms of ERU, there is a growing body of evidence that autoimmune responses to ocular tissue components play a significant role in pathogenesis (6, 11, 38, 39, 40). Cross-reactivity between leptospiral lysates and the cornea or lens has been previously reported (28, 38). Antibodies and T lymphocytes specific for retinal S antigen and interphotoreceptor retinoid binding protein (IRBP) have also been observed in eyes of uveitic horses (6). Moreover, injection of IRBP with complete Freund's adjuvant induced a disease similar to ERU (7). Gilger et al. (11) observed high IL-2 and gamma interferon and low IL-4 mRNA levels in the iris and ciliary body and a predominance of $CD4^+$ Th1 cells in uveitic fluids. They concluded that the intraocular immune response was Th1, most likely associated with delayed-type hypersensitivity (DTH) reactivity to self antigens in the uveal tract. They also observed that peripheral blood lymphocytes of horses with ERU did not exhibit a Th1 response, and they concluded that the local ocular and systemic T-lymphocyte populations were different (11).

Since a proinflammatory DTH response indicates loss of immune privilege, how might intraocular infection by *Leptospira* spp. lead to loss of immune privilege? The results of the present study showed that the local uveitic humoral immune response targets multiple leptospiral proteins. Most, but not all, of these are also strongly recognized by the systemic immune system following abortigenic infections. LruA and LruB are notable because they elicited very strong IgG and IgA responses in uveitic eyes but not in companion sera. Perhaps more significantly, LruA- and LruB-specific antibodies recognized proteins in ocular tissue extracts. Together, the data suggest that LruA and LruB play significant roles in the pathogenesis of ERU. Since immune privilege is permissive of intraocular immune responses that are not proinflammatory, it is possible that the early phase of the immunopathogenesis of ERU involves production of non-complement-fixing antibody and non-DTH T lymphocytes specific for LruA and LruB. Reactivity of these molecules and cells with their targets in ocular tissue then initiates a process that leads to desequestration of IRBP and other ocular autoantigens. Future experiments, including characterization of cross-reacting eye antigens, will explore these hypotheses and lead toward a better understanding of the pathogenesis of leptospiral uveitis.

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